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N-glycosylation in the thermoacidophilic archaeon *Sulfolobus acidocaldarius* involves a short dolichol pyrophosphate carrier

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N-glycosylation is a post-translational modification that occurs across evolution. In the thermoacidophilic archaea, *Sulfolobus acidocaldarius*, glycoproteins are modified by an N-linked tribranched hexasaccharide reminiscent of the N-glycans assembled in Eukarya. Previously, hexose-bearing dolichol phosphate was detected in a *S. acidocaldarius* Bligh–Dyer lipid extract. Here, we used a specialized protocol for extracting lipid-linked oligosaccharides to detect a dolichol pyrophosphate bearing the intact hexasaccharide, as well as its biosynthetic intermediates. Furthermore, evidence for N-glycosylation of two *S. acidocaldarius* proteins by the same hexasaccharide and its derivatives was collected. These findings thus provide novel insight into archaeal N-glycosylation.

Keywords: archaea; dolichol; N-glycosylation; *Sulfolobus acidocaldarius*; thermoacidophile

N-glycosylation, namely the covalent linkage of glycans to select Asn residues of a target protein, is a post-translational modification performed across evolution. Nonetheless, domain-specific differences exist in terms of the composition and architecture of the N-linked glycans, the enzymes used to assemble and attach these glycans and the nature of the phosphorylated polyprenoid lipid carriers upon which N-linked glycans are initially assembled [1–4]. Moreover, it is becoming increasingly clear that archaeal N-glycosylation presents more diversity in each of these aspects of the process than seen in the parallel eukaryal or bacterial pathways [5,6].

Abbreviations

DolP, dolichol phosphate; DolPP, dolichol pyrophosphate; LC-ESI MS, liquid chromatography-electrospray ionization mass spectrometry; MS/MS, tandem mass spectrometry; S-layer, surface layer.

In yeast and higher Eukarya, a dolichol pyrophosphate (DolPP)-linked heptasaccharide is assembled on the cytoplasmic face of the endoplasmic reticulum membrane. Once translocated across the membrane, seven more sugars are transferred from individual dolichol phosphate (DolP) carriers to yield the final 14-member glycan [7,8]. In contrast, in Bacteria, where N-glycosylation is apparently restricted to delta/epsilon proteobacterial species, simpler glycans are assembled on a cytoplasmically oriented undecaprenol pyrophosphate carrier [9–11]. In Archaea, as in Eukarya, dolichol serves as the lipid carrier upon which N-linked glycans are assembled. While both versions of dolichol

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contain a saturated α -position isoprene subunit, all archaeal dolichols considered to date also present a saturated isoprene at the ω -position of the molecule [12–18]. Furthermore, dolichols in the thermophiles *Archaeoglobus fulgidus*, *Pyrobaculum calidifontis*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, and *Sulfolobus solfataricus* have been shown to include saturated isoprenes at more internal positions [14,17,18].

In addition to invoking dolichols demonstrating variety in terms of the degree of isoprene subunit saturation, variability in dolichol phosphorylation is also seen in Archaea, where N-glycosylation seemingly relies on both DolP and DolPP carriers. In some cases, such as the halophile *Haloferax volcanii* and the methanogen *Methanococcus maripaludis*, species for which N-glycosylation pathways have been delineated, N-linked glycans are assembled from precursors derived from one or more DolP carriers [13,15,19]. In other species, DolP bearing complete or derivatives (i.e., precursors or breakdown products) of N-linked glycans decorating glycoproteins have been reported, including *A. fulgidus*, *Haloferax mediterranei*, *Haloarcula marismortui*, *P. furiosus*, *S. acidocaldarius* and *S. solfataricus* [14,16–18,20]. On the other hand, DolPP-bearing glycans decorating glycoproteins in *Methanothermobacter fervidus*, *P. calidifontis* and *S. solfataricus* have been described [18,21]. In *Halobacterium salinarum*, where the surface (S)-layer glycoprotein is modified by two distinct N-linked glycans, one glycan is assembled on DolP while the other is constructed on DolPP [22,23]. Still, no evidence for the contribution of both DolP and DolPP carriers in the biogenesis of the same N-linked glycan, a scenario that occurs in Eukarya, has been demonstrated in Archaea.

Members of the genus *Sulfolobus* may offer examples of archaeal N-glycosylation systems that combine both DolP- and DolPP-linked sugars or glycans. In both *S. acidocaldarius* and *S. solfataricus*, the N-linked glycan corresponds to a complex tribranched oligosaccharide attached to target Asn residues via a di-N-acetylglucosamine core, reminiscent of the N-linked glycan decorating eukaryal glycoproteins [24–26]. In the case of *S. acidocaldarius*, many of the steps involved in the biogenesis of the N-linked hexasaccharide decorating glycoproteins in this species have been described [6,27]. For instance, based on the similarities of the first three sugars assembled on the lipid carrier in *S. acidocaldarius* and eukaryotes, it is thought that the first steps of *S. acidocaldarius* N-glycosylation resemble the parallel steps of the eukaryal N-glycosylation pathway, where Alg7 and Alg13/14 catalyze the assembly of DolPP-N-acetylglucosamine₂-mannose [4,8,28]. Accordingly, genes encoding homologs of Alg7 and Alg13/14 are

found within the *S. acidocaldarius* genome. Recently, DolPP modified by the hexasaccharide precursor of the N-linked heptasaccharide as well as DolP modified by a hexose were observed in *S. solfataricus* [18]. Likewise, DolP-hexose was previously detected in *S. acidocaldarius* [14]. With these latest findings in mind, the involvement of DolPP-linked glycans in *S. acidocaldarius* N-glycosylation was considered in the present report. Furthermore, evidence for the N-glycosylation of two additional *S. acidocaldarius* proteins is provided.

Materials and methods

Strain and growth

S. acidocaldarius (MW001) [29] were grown at 75 °C in Brock's medium [30], pH adjusted to 3 using sulfuric acid, supplemented with 0.1% (w/v) NZ-amine, 0.2% (w/v) dextrin, and 10 $\mu\text{g}\cdot\text{mL}^{-1}$ uracil, under constant shaking. Cell growth was monitored by measuring optical density at 600 nm.

Isolation of *S. acidocaldarius* DolPP-linked glycans

To isolate DolPP-linked glycans, the protocol described by Kelleher *et al.* [31] was employed, with minor modifications. Frozen *S. acidocaldarius* cells were gently thawed and sonicated on ice at room temperature (2 s on, 5 s off, for a total of 1 min; Vibracell VCX750 ultrasonic cell disrupter, Sonics, Newtown, CT, USA). After sonication, the cell suspension was centrifuged for 30 min at 10 000 rpm in a SW-41 rotor at 4 °C to clear nonbroken cells and other debris. The supernatant was transferred into fresh tubes and centrifuged for an additional 45 min at 36 000 rpm in a SW-41 rotor at 4 °C. The resulting supernatant was removed and the pellet was resuspended in 35 mL homogenization buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8) containing 50 mL $\text{CHCl}_3:\text{CH}_3\text{OH}$ (3 : 2) at 4 °C and homogenized using a Pyrex Potter–Elvehjem tissue grinder (Thomas Scientific). After homogenization, 65 mL cold (4 °C) $\text{CHCl}_3:\text{CH}_3\text{OH}$ (3 : 2) were added and the homogenate was mixed by vigorous shaking before centrifugation (3400 g, 15 min, 4 °C). The resulting clear upper aqueous and lowest organic phases were removed and the middle (solid) phase was resuspended in 75 mL of $\text{CHCl}_3:\text{CH}_3\text{OH}$ (3 : 2) containing 1 mM MgCl_2 at room temperature. After vigorous rehomogenization, the suspension was adjusted to a total volume of 150 mL and centrifuged for 15 min (3400 g, 4 °C). The supernatant was removed and the pellet was suspended in 150 mL CH_3OH containing 4 mM MgCl_2 before centrifugation (3400 g, 15 min, 4 °C). These steps were repeated and the resulting

pellet was suspended in 150 mL $\text{CHCl}_3\text{:CH}_3\text{OH:DDW}$ (10 : 10 : 3) and centrifuged in a swing-out rotor (1000 rpm) for 15 min at 22 °C. The supernatant was removed and stored while the pellet were re-extracted with 100 mL $\text{CHCl}_3\text{:CH}_3\text{OH:DDW}$ (10 : 10 : 3) at 37 °C and centrifuged as above. The supernatants obtained from the first and second extractions were combined and the ensuing solution was evaporated at 30 °C. Thereafter, any remaining solvents were removed using a stream of nitrogen. The dried extracts were then subjected to analysis by liquid chromatography coupled with mass spectrometry.

Liquid chromatography-electrospray ionization mass spectrometry (LC-ESI MS) analysis of *S. acidocaldarius* DolPP-linked glycans

Normal phase LC-ESI MS of the *S. acidocaldarius* DolPP-glycan-containing extract was performed using an Agilent 1200 Quaternary LC system coupled to a high-resolution TripleTOF5600 mass spectrometer (Sciex, Framingham, MA, USA). A Unison UK-Amino column (3 μm , 25 cm \times 2 mm) (Imtakt USA, Portland, OR, USA) was used. Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800 : 195 : 5, v/v/v). Mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide (600 : 340 : 50 : 5, v/v/v/v). Mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450 : 450 : 95 : 5, v/v/v/v). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The total LC flow rate was 300 $\mu\text{L}\cdot\text{min}^{-1}$. The post-column splitter diverted ~10% of the LC flow to the ESI source of the TF5600 mass spectrometer, with MS settings as follows: Ion spray voltage (IS) = -4500 V, Curtain gas (CUR) = 20 psi, Ion source gas 1 (GS1) = 20 psi, Declustering potential (DP) = -55 V, and Focusing Potential (FP) = -150 V. Nitrogen was used as the collision gas for tandem mass spectrometry (MS/MS) experiments. Data acquisition and analysis were performed using ANALYST TF1.5 software (Sciex).

Isolation of *S. acidocaldarius* FlaB and SlaB

For isolation of the *S. acidocaldarius* surface (S)-layer that includes SlaB, cell pellets of freshly grown cells (100 mL culture of OD_{600} 0.8) were resuspended in 35 mL buffer A (10 mM NaCl, 0.5% (w/v) *N*-lauroylsarcosine, pH 5.5). The mixture was incubated for 30 min in a rotary shaker at 45 °C, complemented with a small amount of DNase. Subsequent centrifugation at 5000 *g* for 20 min resulted in a

brownish pellet with a white layer (the S-layer) on top. The S-layer was carefully removed and transferred to a 1.5-mL reaction tube, resuspended in 1 mL buffer A and incubated at 45 °C for 20 min under constant shaking. Samples were then centrifuged at maximum speed for 10 min in a tabletop centrifuge and the supernatant was discarded. Repetitive washing with buffer A together with incubation at 45 °C resulted in a white S-layer pellet, free of contaminants. The S-layer was resuspended in buffer A and stored at 4 °C. Before loading onto a SDS/PAGE gel, the S-layer, comprising SlaA and SlaB, was diluted twofold with sodium carbonate buffer (12 mM Na_2CO_3 , pH 10) and incubated at 42 °C for 2 h.

To isolate the archaeellin FlaB, exponentially growing cells were transferred to Brock's medium lacking NZ-amine and dextrin. After starving the cells overnight, the culture was harvested and resuspended in 35 mL Brock's medium without NZ-amine and dextrin. The cell suspension was pumped 10 times through a 0.8-mm diameter injection needle on ice using a peristaltic pump (Gilson/Abimed Minipuls 3). Next, the cells were pumped through an injection needle (0.4-mm diameter) ten times. Samples were centrifuged at 4500 *g* for 20 min at 4 °C. The supernatant was subjected to another round of centrifugation. The cleared supernatant was centrifuged at 240 000 *g* for 12 h at 4 °C to pellet the archaeella, containing FlaB.

LC-ESI MS analysis of *S. acidocaldarius* FlaB and SlaB

For LC-ESI MS analysis of *S. acidocaldarius* FlaB and SlaB, the fractions containing these proteins were separated on 11% polyacrylamide gels and stained with Coomassie R-250 (Roth). For in-gel digestion, the individual protein bands were excised, destained in 400 μL of 50% (vol/vol) acetonitrile (Sigma) in 40 mM NH_4HCO_3 , pH 8.4, dehydrated with 100% acetonitrile, and dried using a SpeedVac drying apparatus. The proteins were reduced with 10 mM dithiothreitol (Sigma) in 40 mM NH_4HCO_3 at 56 °C for 60 min and then alkylated for 45 min at room temperature with 55 mM iodoacetamide in 40 mM NH_4HCO_3 . The gel pieces were washed with 40 mM NH_4HCO_3 for 15 min, dehydrated with 100% acetonitrile, and SpeedVac-dried. The gel slices were rehydrated with 12.5 $\text{ng}\cdot\mu\text{L}^{-1}$ of mass spectrometry (MS)-grade Trypsin Gold (Promega) in 40 mM NH_4HCO_3 . The protease-generated peptides were extracted with 0.1% (v/v) formic acid in 20 mM NH_4HCO_3 , followed by sonication for 20 min at room temperature, dehydration with 50% (v/v) acetonitrile, and additional sonication. After three rounds of extraction, the gel pieces were dehydrated with 100% acetonitrile, dried completely with a SpeedVac, resuspended in 5% (v/v) acetonitrile containing 1% formic acid (v/v) and infused into the mass spectrometer using static nanospray Econotips (New Objective, Woburn, MA). The protein digests were separated on-

line by nano-flow, reverse-phase liquid chromatography by loading onto a 150-mm by 75- μ m (internal diameter) by 365- μ m (external diameter) Jupifer preppacked fused silica 5- μ m C₁₈ 300Å reverse-phase column (Thermo Fisher Scientific, Bremen, Germany). The sample was eluted into the LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) using a 60-min linear gradient of 0.1% formic acid (v/v) in acetonitrile/0.1% formic acid (1 : 19, by volume) to 0.1% formic acid in acetonitrile/0.1% formic acid (4 : 1, by volume) at a flow rate of 300 nL·min⁻¹.

Results

S. *acidocaldarius* contains glycan-charged DolP and DolPP

In earlier attempts at identifying lipid-linked glycans putatively involved in *S. acidocaldarius* N-glycosylation, both dolichol and DolP were identified [14]. The extremely short dolichol was shown to be saturated not only at the α - and ω -positions, as holds true for dolichol in all Archaea considered to date but also at more internal positions. When, however, glycosylated versions of this polyprenoid were sought, only hexose-charged DolP were detected. As such, it was proposed at the time that a different method than the Bligh and Dyer [32] protocol might be needed for extracting such lipids containing higher ordered glycans. Accordingly, in the present study, the extraction protocol developed by Kelleher *et al.* [31] was adopted.

Following such extraction, the [M-H]⁻ ion of hexose-charged C₄₅DolP was identified by LC-ESI MS; its [M-H]⁻ monoisotopic ion peak was observed at *m/z* 881.666 (Fig. 1A), as first described by Guan *et al.* [14]. In addition, a doubly charged ion peak of *m/z* 958.448, corresponding to the monoisotopic [M-2H]²⁻ ion peak of C₄₅DolPP bearing the hexasaccharide N-linked to *S. acidocaldarius* glycoproteins was also detected (Fig. 1B) [24,25]. The deduced monoisotopic mass (1918.912 Da) is in agreement with the calculated mass (1917.917 Da) of C₄₅DolPP linked to a *N*-acetylglucosamine₂-mannose₂-sulfated quinovose-glucose hexasaccharide. The identification was further verified by MS/MS. The product spectrum of the [M-2H]²⁻ ion at *m/z* 958.448 showed peaks consistent with DolPP bearing the previously defined N-linked hexasaccharide decorating *S. acidocaldarius* glycoproteins (Fig. 1C). Specifically fragments corresponding to DolPP and the hexasaccharide were detected.

In addition to hexasaccharide-modified DolPP, the *S. acidocaldarius* lipid extract also included C₄₅DolPP bearing a pentasaccharide comprising *N*-acetylhexosamine₂-hexose₂-sulfated quinovose (Fig. 2A) and a

tetrasaccharide comprising *N*-acetylhexosamine₂-hexose-sulfated quinovose (Fig. 2B). DolPP modified by *N*-acetylhexosamine₂-hexose, *N*-acetylhexosamine₂ or *N*-acetylhexosamine were not detected.

LC-ESI MS analysis of S. *acidocaldarius* glycoproteins reveals novel sites of N-glycosylation

To date, N-glycosylation by a hexasaccharide comprising *N*-acetylhexosamine₂-hexose₃-sulfated quinovose hexasaccharide has been demonstrated for two *S. acidocaldarius* glycoproteins, namely cytochrome b558/566 [24] and SlaA, a component of the surface layer [25]. In addition, site-directed modification of predicted N-glycosylation sites of FlaB, a component of the archaellum, led to changes in the SDS/PAGE migration protein, providing support for it also undergoing such post-translational modification [33]. In the present study, LC-ESI MS was performed on *S. acidocaldarius* glycoproteins to confirm previous assignments, to identify new N-glycosylation targets and to gain additional insight into the process of N-glycosylation in this organism.

The membrane protein SlaB is one of the two glycoproteins comprising the *S. acidocaldarius* S-layer [25]. The LC-ESI MS profile of a SlaB-derived Asn-278-containing peptide generated by trypsin treatment (²⁶⁵VTTAPVSAQVYYPNGTQT²⁸⁴) was considered. Such analysis detected a [M + 2H]²⁺ peak at *m/z* 1062.55 (Fig. 3A, inset). This value is in excellent agreement with the calculated [M + 2H]²⁺ mass of the peptide, 1062.55 Da. The same mass spectrometry profile also included a [M + 2H]²⁺ peak at *m/z* 1621.72 (Fig. 3A), likely corresponding to the same peptide modified by *N*-acetylhexosamine₂-hexose₃-sulfated quinovose (calculated mass 1621.85 Da). In addition, [M + 2H]²⁺ peaks at *m/z* 1164.09 (Fig. S1A), 1265.63 (Fig. S1B), 1346.66 (Fig. S1C), 1427.69 (Fig. S1D), 1459.67 (Fig. S1E), and 1540.69 (Fig. S1F) were observed. These values are in good agreement with the predicted [M + 2H]²⁺ values of the Asn-278-containing peptide, respectively, modified by *N*-acetylhexosamine (calculated mass 1164.09 Da), *N*-acetylhexosamine₂ (calculated mass 1265.63 Da), *N*-acetylhexosamine₂-hexose (calculated mass 1346.70 Da), *N*-acetylhexosamine₂-hexose₂ (calculated mass 1427.77 Da), *N*-acetylhexosamine₂-hexose-sulfated quinovose (calculated mass 1459.71 Da), and *N*-acetylhexosamine₂-hexose₂-sulfated quinovose (calculated mass 1540.78 Da). To confirm this assessment, the [M + 2H]²⁺ base peak at *m/z* 1621.72, thought to correspond to the peptide modified by the complete hexasaccharide, was subjected to MS/MS. The

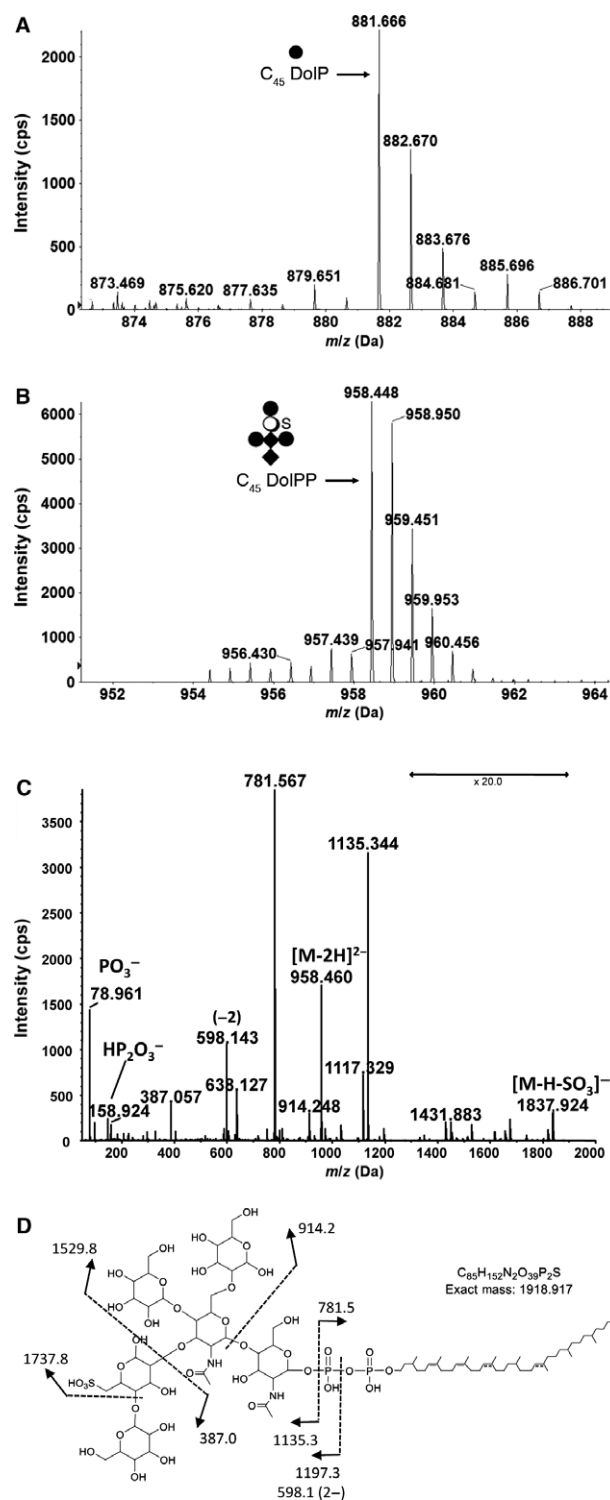


Fig. 1. Glycan-charged phosphorylated dolichol species in *S. acidocaldarius*. LC-ESI MS analysis of a *S. acidocaldarius* lipid extract revealed the presence of (A) C_{45} DolP modified by a hexose and (B) C_{45} DolPP modified by a hexasaccharide comprising *N*-acetylhexosamine₂-hexose₃-sulfated quinovose (QuiS). (C) MS/MS spectrum of $[M-2H]^{2-}$ at m/z 958.4. (D) Fragmentation scheme for the observed product ions in C. The depicted DolPP-linked glycan structure is based on the previously reported protein N-linked glycan [24,25]. The arrows indicating $\times 20$ reflect magnification of the ion peaks in the corresponding region of the m/z values on the graph. Where the position of a double bond is only speculated, a dotted line is drawn. Diamonds represent *N*-acetylhexosamines, full circles represent hexoses, open circles represent quinovose and S represents sulfation.

In addition, a trypsin-generated peptide containing both Asn-425 and Asn-430 was shown to be modified at only one of these positions, although it was not possible to determine which. Thus, of 13 potential N-glycosylation sites in SlaB, five were determined as being modified and one was deemed as not being processed.

In the case of FlaB, the filament protein of the archaeallum, the archaeal motility structure [34], direct evidence for modification of one of the six putative N-glycosylation sites in the protein by the same hexasaccharide was provided. LC-ESI MS analysis of an Asn-195-containing tryptic peptide ($^{182}GGQLTSSPLYIISNTSIVASKPWLK^{206}$) revealed a $[M+3H]^{3+}$ peak at m/z 887.50, a value in excellent agreement with the calculated $[M+3H]^{3+}$ mass of the peptide, 887.49 Da (Fig. 4A, inset). A $[M+3H]^{3+}$ peak at m/z 1260.27 was also observed (Fig. 4A), likely corresponding to the same peptide modified by *N*-acetylhexosamine₂-hexose₃-sulfated quinovose (calculated mass 1260.36 Da). The same mass spectrometry profile also contained $[M+3H]^{3+}$ peaks at m/z 955.19, likely corresponding to the peptide modified by *N*-acetylhexosamine (calculated mass 955.18 Da) (Fig. S2A), at m/z 1022.88, likely corresponding to the peptide modified by *N*-acetylhexosamine₂ (calculated mass 1022.87 Da) (Fig. S2B), at m/z 1076.90, likely corresponding to the peptide modified by *N*-acetylhexosamine₂-hexose (calculated mass 1076.90 Da) (Fig. S2C), at m/z 1130.92, likely corresponding to the peptide modified by *N*-acetylhexosamine₂-hexose₂ (calculated mass 1130.97 Da) (Fig. S2D), at m/z 1152.24, likely corresponding to the peptide modified by *N*-acetylhexosamine₂-hexose-sulfated quinovose (calculated mass 1152.26 Da) (Fig. S2E), and at m/z 1206.26, likely corresponding to the peptide modified by *N*-acetylhexosamine₂-hexose₂-sulfated quinovose (calculated mass 1206.31 Da) (Fig. S2F). MS/MS analysis of the $^{13}C_1$ -containing isotopic $[M+2H]^{2+}$ peak at m/z 1890.291, thought to correspond to the peptide modified by the

fragmentation pattern obtained was consistent with this previously described N-linked glycan (Fig. 3B). Experimental evidence for the similar modification of SlaB Asn-357, Asn-376, and Asn-397 was also obtained (Table 1).

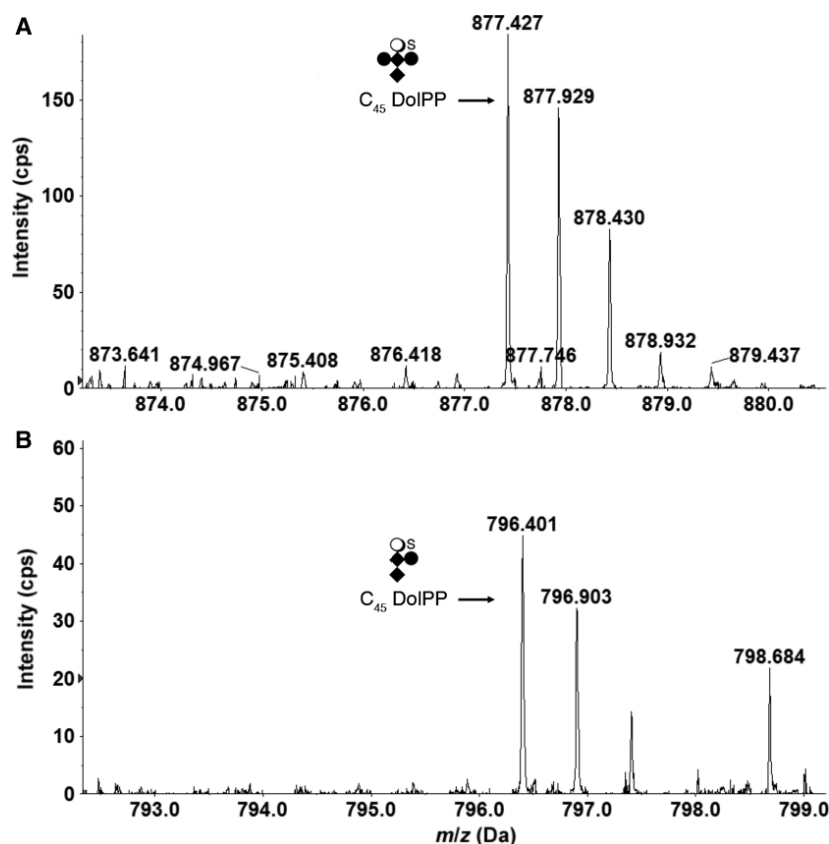


Fig. 2. Glycan-charged *S. acidocaldarius* DolPP species. (A) C₄₅ DolPP modified by a pentasaccharide comprising *N*-acetylhexosamine₂-hexose₂-sulfated quinovose. (B) C₄₅ DolPP modified by a tetrasaccharide comprising *N*-acetylhexosamine₂-hexose-sulfated quinovose. Diamonds represent *N*-acetylhexosamines, full circles represent hexoses, open circles represent quinovose, and S represents sulfation.

14

complete hexasaccharide, yielded a fragmentation pattern consistent with these predictions (Fig. 4B).

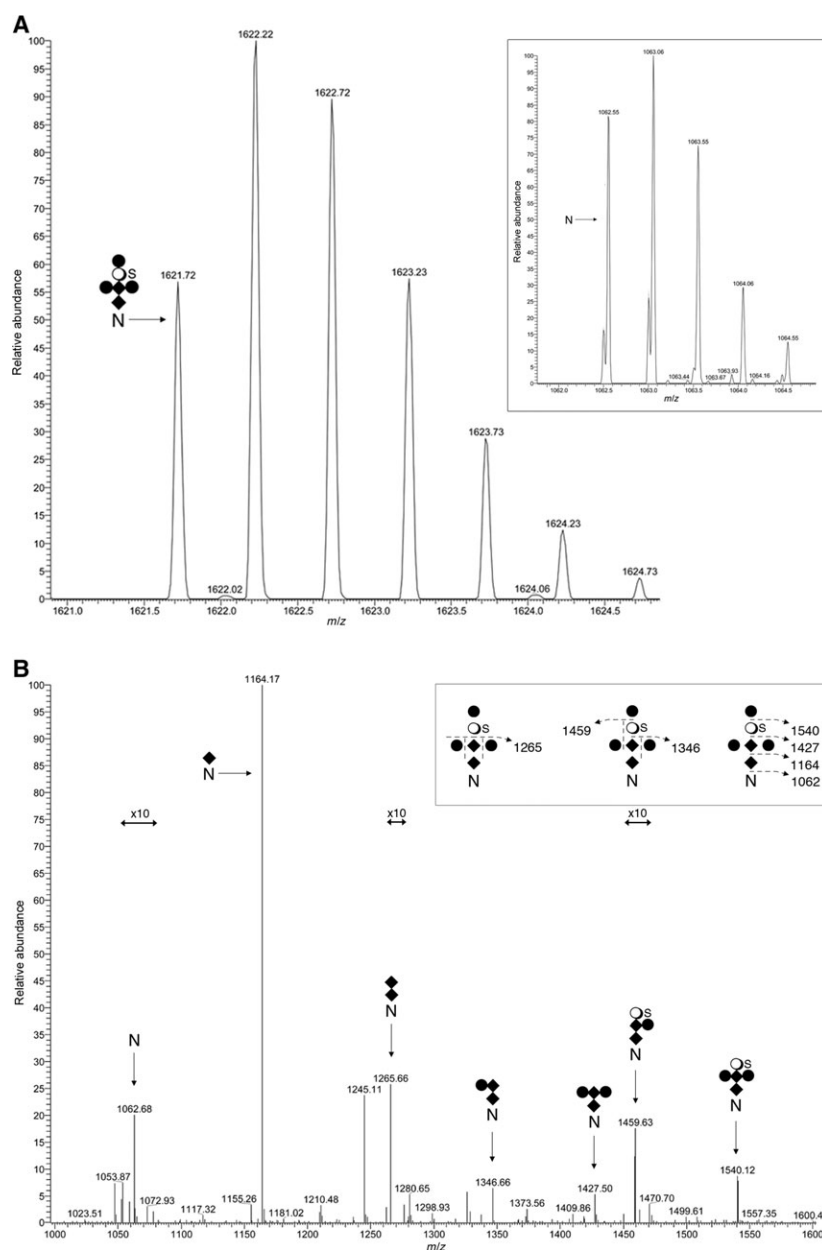
Discussion

When compared to the considerable body of data available on eukaryal and bacterial N-glycosylation, the archaeal version of this post-translational modification remains poorly understood. In recent years, however, considerable progress in closing this gap has been realized. Today, information is available on a number of N-linked glycan structures, on enzymes involved in the biosynthesis of these glycans, and on the lipids upon which these glycans are assembled (for recent review, see ref. [6]). Still, concerted efforts to describe all of these aspects of the N-glycosylation process in a single archaeal species have thus far focused on a limited number of organisms, namely *Hfx. volcanii*, *M. maripaludis* and *S. acidocaldarius*. In the present study, novel insight into *S. acidocaldarius* N-glycosylation was obtained.

A major finding of this study was that the complete hexasaccharide N-linked to *S. acidocaldarius* glycoproteins is assembled on a single DolPP carrier, prior to its transfer to target Asn residues. This apparently

differs from the situation in *S. solfataricus*, where it was reported that only the hexasaccharide precursor of the N-linked heptasaccharide, was detected as DolPP-bound, with the seventh sugar being possibly derived from a DolP carrier, perhaps after delivery of the hexasaccharide to target Asn residues [18]. If so, then the role played by the DolPP-bound hexose detected in earlier studies of *S. acidocaldarius* is unclear [14]. It is possible that in *S. acidocaldarius*, DolP-hexose provides one or more hexoses to the growing DolPP-linked hexasaccharide. Alternatively, DolP-hexose may participate in glycolipid biogenesis in this organism. Indeed, *S. acidocaldarius* membranes contain lipids bearing phosphomyoinositol, glucopyranose, galactylpyranosyl-glucopyranose [35].

It was further shown that the hexasaccharide N-linked to FlaB and SlaB is identical to that previously reported to decorate SlaA and cytochrome b558/566 [24,25]. Although DolPP-bearing glycans comprising less than four of the sugars comprising the N-linked hexasaccharide sugars was not detected, assessing FlaB- and SlaB-derived glycopeptides nonetheless offered novel insight into the *S. acidocaldarius* N-glycosylation pathway. The detection of proteins bearing various precursors of the N-linked hexasaccharide



argues that DolPP bearing such precursors can be processed by AglB, the oligosaccharyltransferase responsible for transferring glycans from DolPP carriers to target Asn residues, as well as by the currently unidentified flippase responsible for translocating glycan-charged DolPP across the membrane. The fact that certain derivatives of the N-linked hexasaccharide, such as *N*-acetylglucosamine₂-sulfoquinovose, were not detected argues that these glycopeptides bore precursors rather than breakdown products of the hexasaccharide. As such, and in agreement with previous reports [25], the results presented here show that the

two *N*-acetylglucosamines of the N-linked hexasaccharide are first loaded onto the DolPP carrier, followed by a mannose. The fact that the glycopeptides bearing both *N*-acetylglucosamine₂-mannose-sulfoquinovose and *N*-acetylglucosamine₂-mannose₂ were detected argues that *S. acidocaldarius* can add both sulfoquinovose and mannose to the DolPP-linked trisaccharide precursor, although the present results cannot determine which of the lipid-linked tetrasaccharides is further processed or whether both are. However, earlier work involving the deletion of the glycosyltransferase-encoding *agl3* gene supported *N*-acetylglucosamine₂-mannose-

Table 1. N-glycosylated positions in *S. acidocaldarius* FlaB and SlaB, as identified by LC-ESI MS.

Protein	Peptide	Observed mass (<i>m/z</i>)	Calculated mass (<i>m/z</i>)	Glycan
FlaB	GGQLTSSPLYIIS ¹⁹⁵ NTSIVASKPWLK	1260.27 ³⁺	1260.36	HexNAc ₂ Hex ₃ QuiS
		1206.26 ³⁺	1206.31	HexNAc ₂ Hex ₂ QuiS
		1152.24 ³⁺	1152.26	HexNAc ₂ HexQuiS
		1130.92 ³⁺	1130.97	HexNAc ₂ Hex ₂
		1076.90 ³⁺	1076.92	HexNAc ₂ Hex
		1022.88 ³⁺	1022.87	HexNAc ₂
		955.19 ³⁺	955.18	HexNAc
SlaB	VTTAPVSAQVYYP ²⁷⁸ NGTQTVK	1621.72 ²⁺	1621.85	HexNAc ₂ Hex ₃ QuiS
		1540.69 ²⁺	1540.78	HexNAc ₂ Hex ₂ QuiS
		1459.67 ²⁺	1459.71	HexNAc ₂ HexQuiS
		1427.69 ²⁺	1427.77	HexNAc ₂ Hex ₂
		1346.66 ²⁺	1346.70	HexNAc ₂ Hex
		1265.63 ²⁺	1265.63	HexNAc ₂
		1164.09 ²⁺	1164.09	HexNAc
	EISSLVQ ³⁵⁷ NITNLEK	1353.60 ²⁺	1353.73	HexNAc ₂ Hex ₃ QuiS
		1272.57 ²⁺	1272.66	HexNAc ₂ Hex ₂ QuiS
		1191.55 ²⁺	1191.59	HexNAc ₂ HexQuiS
		1159.57 ²⁺	1159.65	HexNAc ₂ Hex ₂
		1078.54 ²⁺	1078.58	HexNAc ₂ Hex
		997.51 ²⁺	997.51	HexNAc ₂
		895.97 ²⁺	895.97	HexNAc
	TVNNLQTOISTL ³⁷⁶ NSSLSSLSQR	1170.53 ³⁺	1170.53	HexNAc ₂ Hex ₃ QuiS
		1116.51 ³⁺	1116.57	HexNAc ₂ Hex ₂ QuiS
		1062.50 ³⁺	1062.52	HexNAc ₂ HexQuiS
		1041.18 ³⁺	1041.18	HexNAc ₂ Hex ₂
		987.16 ³⁺	987.18	HexNAc ₂ Hex
		933.11 ³⁺	933.13	HexNAc ₂
		865.45 ³⁺	865.45	HexNAc
	ITSLQNTLASL ³⁹⁷ NSTISSLSGTASSLSSQLNALQK	1537.73 ³⁺	1537.82	HexNAc ₂ Hex ₃ QuiS
		1483.71 ³⁺	1483.77	HexNAc ₂ Hex ₂ QuiS
		1429.67 ³⁺	1429.72	HexNAc ₂ HexQuiS
		n.d.	1408.43	HexNAc ₂ Hex ₂
		1354.36 ³⁺	1354.38	HexNAc ₂ Hex
		1300.33 ³⁺	1300.33	HexNAc ₂
		1232.66 ³⁺	1232.64	HexNAc
	ISSL ⁴²⁵ <u>NSSIT</u> ⁴³⁰ <u>NLSGQLNSLQSK</u> *	1103.84 ³⁺	1103.93	HexNAc ₂ Hex ₃ QuiS
		1049.82 ³⁺	1049.88	HexNAc ₂ Hex ₂ QuiS
		995.80 ³⁺	995.83	HexNAc ₂ HexQuiS
		n.d.	974.54	HexNAc ₂ Hex ₂
		920.46 ³⁺	920.49	HexNAc ₂ Hex
		866.44 ³⁺	866.43	HexNAc ₂
		798.75 ³⁺	798.75	HexNAc

n.d. – not detected.

*Asn-425 or Asn-430 is modified but not both.

sulfoquinovose as being the precursor of the hexasaccharide [36]. This scenario is reminiscent of what occurs in *Hfx. volcanii*, where two different trisaccharide-modified DoIP species are seemingly generated, with only one serving as the precursor of the N-linked pentasaccharide and the other not undergoing further processing before being delivered to target Asn residues [37].

Still, the detection of hexasaccharide-charged DoIPP raises new questions concerning the *S. acidocaldarius*

N-glycosylation pathway. For instance, it remains to be determined whether DoIPP is charged with *N*-acetylglucosamine or whether a phosphorylated version of the sugar is added to a DoIP carrier to yield DoIPP bearing this first subunit of the hexasaccharide. Accordingly, *S. acidocaldarius aglH* is a homolog of eukaryal *agl7*, encoding a UDP- *N*-acetylglucosamine: DoIP *N*-acetylglucosamine-1-phosphotransferase [38]. Although *S. acidocaldarius aglH* could not be deleted,

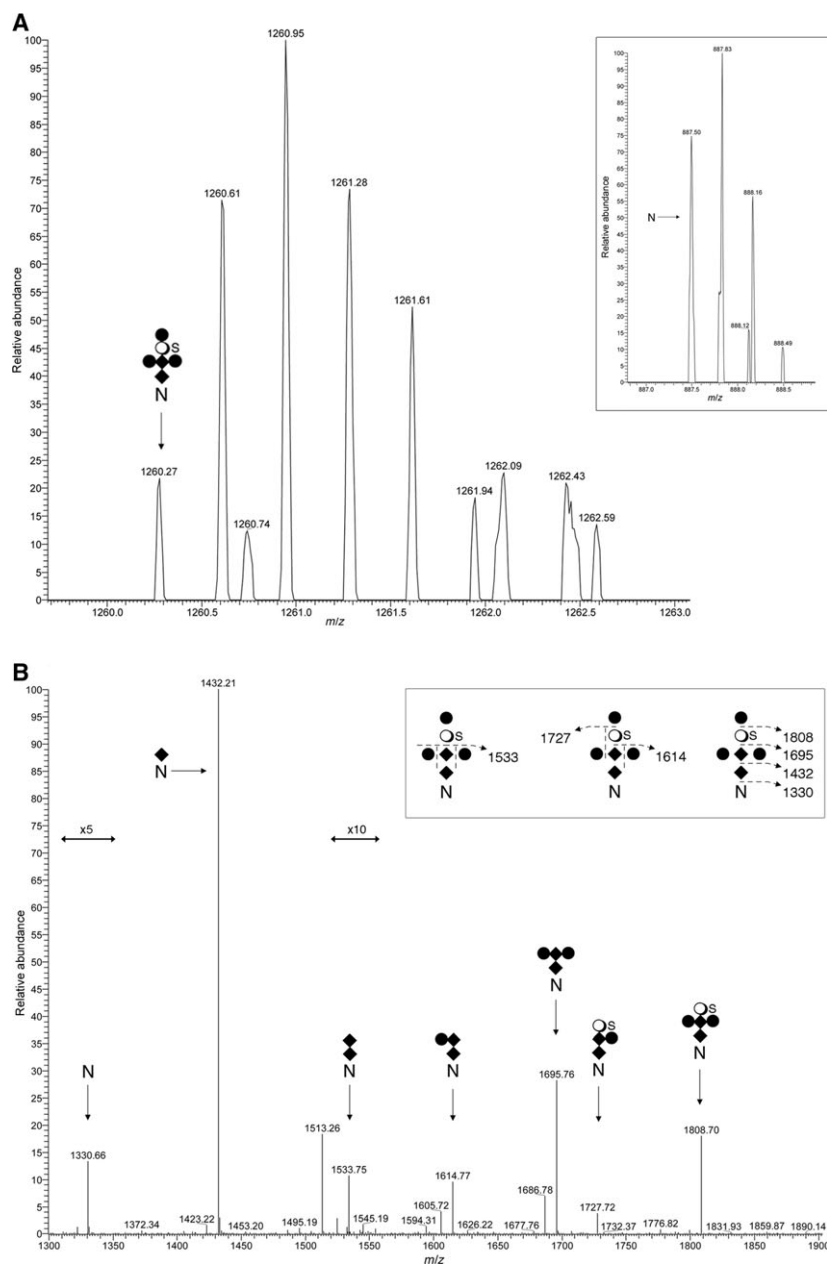


Fig. 4. N-glycosylation of *S. acidocaldarius* FlaB. (A) LC-ESI MS analysis of a tryptic fragment of FlaB that includes Asn-195 revealed the peptide (inset) and its modification by a hexasaccharide comprising *N*-acetylhexosamine₂-hexose₃-sulfated quinovose. $[M + 3H]^{3+}$ ion peaks are presented. (B) MS/MS profile of the $^{13}C_1$ -containing isotopic $[M + 2H]^{2+}$ peak at m/z 1890.291. The inset schematically represents the fragmentation scheme. The N represents Asn-195, diamonds represent *N*-acetylhexosamines, full circles represent hexoses, open circles represent quinovose and S represents sulfation. The arrows indicating $\times 5$ and $\times 10$ reflect magnification of the ion peaks in the corresponding region of the m/z values on the graph.

the introduced gene could rescue a yeast *agl7* conditional mutant (B.H. Meyer and S.V. Albers, unpublished). This argues for DoIP being charged with nucleotide-activated *N*-acetylglucosamine to yield DoIPP-*N*-acetylglucosamine. At the same time, when the same experiment was performed using *Methanococcus voltae aglH*, where DoIP is thought to be the lipid glycan carrier [15], complementation of the same yeast mutant was realized [39].

With glycan-charged lipid carriers, N-linked glycan composition, sites of modification in reporter glycoproteins, and several enzymes participating in glycan assembly and attachment already known, together

with gene deletion techniques and mass spectrometry protocols that will allow for the identification of additional pathway components, the stage is now set for detailed delineation of the *S. acidocaldarius* N-glycosylation process.

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Author contributions

ZG, AD, PN, and BM performed the experiments; ZG, AD, SVA, and JE analyzed the data; JE wrote the manuscript.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. N-glycosylation of *S. acidocaldarius* SlaB.

Fig. S2. N-glycosylation of *S. acidocaldarius* FlaB.